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Activation of PI3K/Akt and MAPK pathways regulates Myc-mediated transcription by phosphorylating and promoting the degradation of Mad1

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Mad1, a member of the Myc/Max/Mad family, suppresses Myc-mediated transcriptional activity by competing with Myc for heterodimerization with its obligatory partner, Max. The expression of Mad1 suppresses Myc-mediated cell proliferation and transformation. The levels of Mad1 protein are generally low in many human cancers, and Mad1 protein has a very short half-life. However, the mechanism that regulates the turnover of Mad1 protein is poorly understood. In this study, we showed that Mad1 is a substrate of p90 ribosomal kinase (RSK) and p70 S6 kinase (S6K). Both RSK and S6K phosphorylate serine 145 of Mad1 upon serum or insulin stimulation. Ser-145 phosphorylation of Mad1 accelerates the ubiquitination and degradation of Mad1 through the 26S proteasome pathway, which in turn promotes the transcriptional activity of Myc. Our study provides a direct link between the growth factor signaling pathways regulated by PI3 kinase/Akt and MAP kinases with Myc-mediated transcription.

phosphorylation | RSK | S6K1 | ubiquitination | proliferation

Mad1 (encoded by the gene *mxd1*), a member of the Myc/Mad/Max family known as bHLH/LZip proteins (1, 2), is recognized as an important cellular antagonist of Myc (1, 2). Mad1 antagonizes Myc function by recruiting Max and the mSin3 repressor complex to Myc-responsive elements, directly competing with the Myc–Max dimer for access to transcriptional sites (1, 2). The half-life of Mad1 protein, like that of Myc protein, is very short (1), and the levels of Mad1 are tightly regulated during cell proliferation and differentiation (2). In contrast, the levels of Max protein were found to be constitutive under different conditions (3, 4). Therefore, because both Myc and Mad1 compete for Max (1), the availability of Max to Myc is profoundly dependent on the expression levels of Mad1 protein (and vice versa).

PtdIns3 kinase (PI3K) is activated in response to mitogen stimulation of multiple receptor tyrosine kinases and G protein-coupled receptors. PI3K phosphorylates phosphatidylinositol-4, 5-bisphosphate (PIP2) to generate phosphatidylinositol-3,4, 5-trisphosphate (PIP3) (5). PIP3 is critical in mediating the activation of a number of important downstream effector molecules, including Ser/Thr protein kinase Akt/PKB, which further activates mammalian target of rapamycin (mTOR). mTOR in turn propagates the signals by its two important downstream effectors, the eukaryotic initiation factor 4E (eIF4E) and the p70 ribosomal S6 kinase 1 (S6K1). eIF4E and S6K1 mediate mTOR-dependent cell size control (6), as well as contribute to efficient G_1 cell cycle progression (7).

A number of observations suggest that Myc specifically cooperates with activated PI3K in deregulated cell proliferation and transformation. For example, coexpression of c-Myc and PI3K was found to be sufficient to transform early passage human mammary epithelial cells and fibroblasts *in vitro* in the presence of hTERT and SV40 large T (8). This cooperation has been shown to occur indirectly by regulating downstream target gene transcription. For example, the phosphorylation of FoxO transcription factors by Akt has been shown to alleviate the inhibition of Myc target genes by FoxO transcriptional factors and to facilitate Myc-mediated transcription and cellular transformation (9). However, no direct interaction of the PI3K pathway with Myc/Max/Mad family of proteins has been described.

In this study, we show that the levels of Mad1 are rapidly decreased upon mitogen stimulation, and this degradation is regulated by the MAPK and PI3K/Akt pathways. We provide evidence that both RSK and S6K1 phosphorylate Mad1 and phosphorylation of Mad1 accelerates its ubiquitination and proteasome-mediated degradation, thereby inhibiting the tumor suppressor function of Mad1. Our findings provide a novel mechanism by which the growth factor signaling pathways cooperate with Myc to promote proliferation and cellular transformation.

Results

The Cellular Levels of Mad1 Are Rapidly Reduced upon Mitogen or Insulin Stimulation. Because Myc and Mad1 have been shown to antagonistically regulate G_0–S progression and the levels of Myc proteins are increased during G_0–S transition (10, 11), we examined the abundance of Mad1 during cell cycle reentry after starvation. HeLa cells were made quiescent by serum starvation for 48 h and then reactivated by the addition of serum. Interestingly, the levels of nuclear Mad1 were increased in serum-starved cells, but rapidly reduced upon serum stimulation (Fig. 1*A*). Our previous results show Mad1 is exclusively in nuclei even in the presence of the proteasome inhibitor MG132 (12). This reduction of nuclear Mad1 levels, however, was not due to nuclear export because Mad1 was not detected in the cytosolic fractions in serum-stimulated cells (Fig. 1*A*). To test whether the reduction of Mad1 upon serum stimulation was due to increased proteasomal degradation, we pretreated quiescent HeLa cells with MG132 for 1 h before serum addition. Treatment of MG132 prevented the disappearance of nuclear Mad1 upon serum addition (Fig. 1*B*), suggesting that the reduction of Mad1 levels in serum-stimulated cells was due to proteasomal degradation.

To further exclude the possibility that the reduction of Mad1 levels was due to transcriptional regulation, we generated a stable HeLa cell line expressing Flag-tagged Mad1 (HeLa-Flag-Mad1) driven by the murine phosphoglycerate kinase (PGK) promoter. This polyclonal stable cell line was made by infecting HeLa cells with virus expressing Flag-tagged Mad1 and selected surviving cells as a pool. Similar to those of endogenous Mad1,

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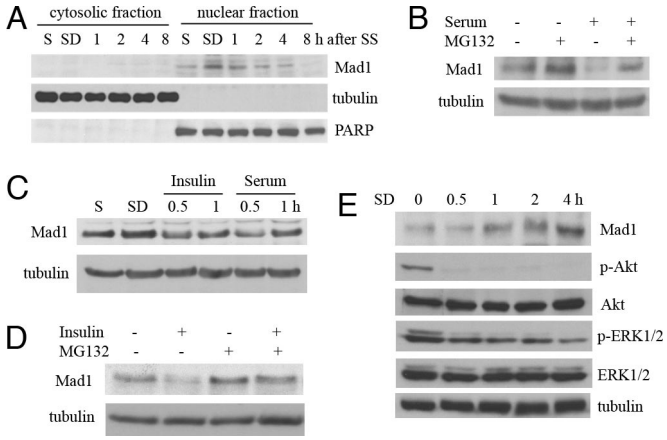

Fig. 1. The cellular levels of Mad1 are rapidly reduced upon mitogen or insulin stimulation. (*A*) HeLa cells were cultured in the presence (S) or absence of serum (SD) for 48 h before stimulation with serum (SS) for the indicated amount of time. The lysates were fractionated and analyzed by Western blotting with the indicated antibodies. (*B*) HeLa cells were starved for 48 h and pretreated with DMSO or MG132 (10 μM) for 1 h before serum stimulation for 3 h. The Mad1 levels were analyzed by Western blotting. (*C*) The experiment was performed as in *A* except by using HeLa-Flag-Mad1 cells and stimulating with insulin (10 μg/ml) as indicated. (*D*) HeLa-Flag-Mad1 cells were starved overnight and pretreated with DMSO or MG132 (10 μM) for 1 h before stimulation with insulin (10 μg/ml) for 30 min. The Mad1 levels were analyzed by Western blotting. (*E*) HeLa cells were starved (SD) with serum-free medium for indicated amount of time. The cell lysates were analyzed by using Western blotting with the indicated antibodies.

serum stimulation also down-regulated the levels of Flag-tagged Mad1 (Fig. 1*C*). Furthermore, the addition of insulin (10 μg/ml) to quiescent HeLa-Flag-Mad1 cells also reduced the levels of Flag-tagged Mad1, which was inhibited by the presence of MG132 (Fig. 1 *C* and *D*). Because serum or high concentration of insulin (10 μg/ml) stimulation activates both the PI3K/Akt and MAPK pathways, we hypothesized that the activation of PI3K/Akt or MAPK may be involved in mediating the degradation of Mad1. Consistent with this possibility, the endogenous Mad1 levels were rapidly increased in serum-starved HeLa cells, and correlated with the dephosphorylation of Akt and ERK (Fig. 1*E*).

AGC Kinases Phosphorylate Mad1 *in Vitro* and in Cells. Because Mad1 protein demonstrated reduced protein mobility on SDS-page electrophoresis in cells stimulated with insulin in the presence of MG132 (Fig. 1*D*), we hypothesized that Mad1 may be phosphorylated as a result of PI3K/Akt or MAPK pathway activation. To test this hypothesis, we analyzed the Mad1 protein sequence using the Scansite (http://scansite.mit.edu) and found a conserved consensus phosphorylation site (RXRXXS) in Mad1 for the AGC kinase family, which includes Akt, S6K1, and RSK (Fig. 2*A*). Using an *in vitro* kinase assay, we found that both S6K1 and RSK could robustly phosphorylate purified GST-Mad1, but not GST alone (Fig. 2*B*). The phosphorylation was largely abolished in Mad1 S145A mutant, suggesting that Ser-145 is a major site of S6K1 and RSK phosphorylation in Mad1 (Fig. 2*B*). Similarly, Akt could also phosphorylate purified GST-Mad1 mainly on Ser-145 (Fig. 2*C*). These results indicated that all of the three AGC kinases tested, Akt, RSK, and S6K, can phosphorylate Mad1 *in vitro*.

We then tested whether overexpression of the three AGC kinases leads to Mad1 phosphorylation in cells. The phosphorylation of Mad1 at Ser-145 was monitored by Western blot analysis with an anti-phospho-S145 Mad1-specific antibody. As shown in Fig. 2*D*, overexpression of a wild-type allele of S6K1 led

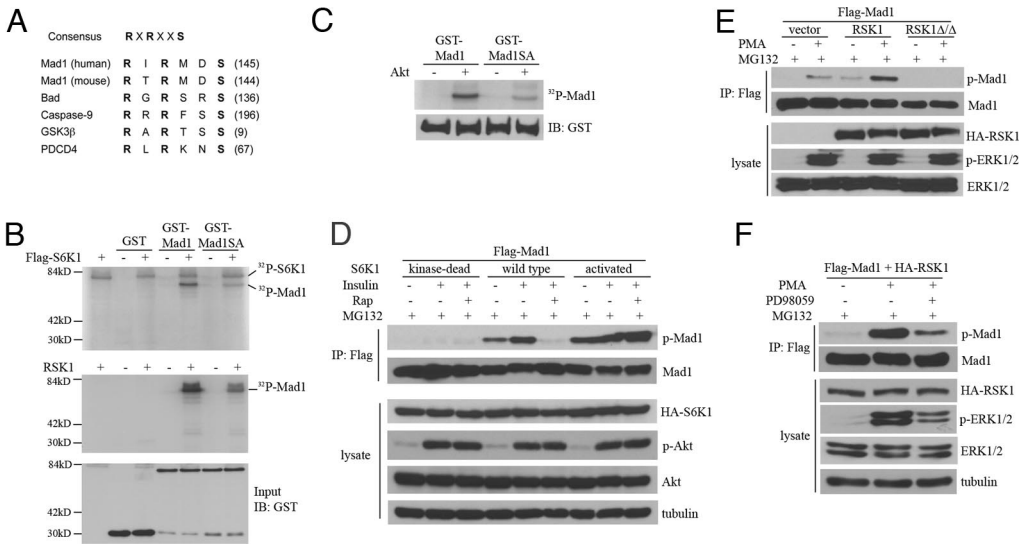

Fig. 2. AGC kinases phosphorylates Mad1 *in vitro* and in cells. (*A*) Alignment of the amino acid sequences of the putative AGC kinase phosphorylation site in Mad1 and previously reported AGC kinase substrates. (*B*) 293T cells were transfected with expression vector of Flag-S6K1. Cells were stimulated with insulin (10 μg/ml) for 15 min. Activated Flag-S6K1 was immunoprecipitated from the cell lysate by using anti-Flag M2 beads and was eluted with Flag peptide. Purified GST, GST-Mad1, or GST-Mad1S145A protein (1 μg) was incubated with activated Flag-S6K1 or recombinant RSK1 in the presence of [γ-^{32}P] ATP. ^{32}P-labeled proteins were resolved by SDS/PAGE and visualized by autoradiography. The input of GST proteins was shown by Western blot analysis with anti-GST antibody. (*C*) The experiment was performed as in *B* except by using recombinant activated Akt. (*D*) 293T cells were transfected with vectors encoding Flag-tagged Mad1 with kinase-dead, wild-type, or activated alleles of S6K1. Cells were serum-starved for 18 h, pretreated with MG132 (10 μM) and with or without rapamycin (Rap; 20 nM) for 1 h and stimulated with insulin (0.5 μg/ml) for 15 min. Flag-Mad1 was immunoprecipitated and immunoblotted with anti-phospho-Mad1 and anti-Mad1 antibodies. Total cell extracts were analyzed by Western blotting with the indicated antibodies. (*E*) 293T cells were transfected with vectors encoding Flag-tagged Mad1 wild-type or kinase-dead RSK1. After serum starvation for 18 h, cells were treated with MG132 (10 μM) for 1 h and stimulated with PMA (50 nM) for 15 min. Analysis was performed as in *D*. (*F*) 293T cells were transfected with vectors encoding Flag-tagged Mad1 and wild-type RSK1. After serum starvation for 18 h, cells were treated with MG132 (10 μM) and with or without PD98059 (50 μM) for 1 h and stimulated with PMA (50 nM) for 15 min. Analysis was performed as in *D*.

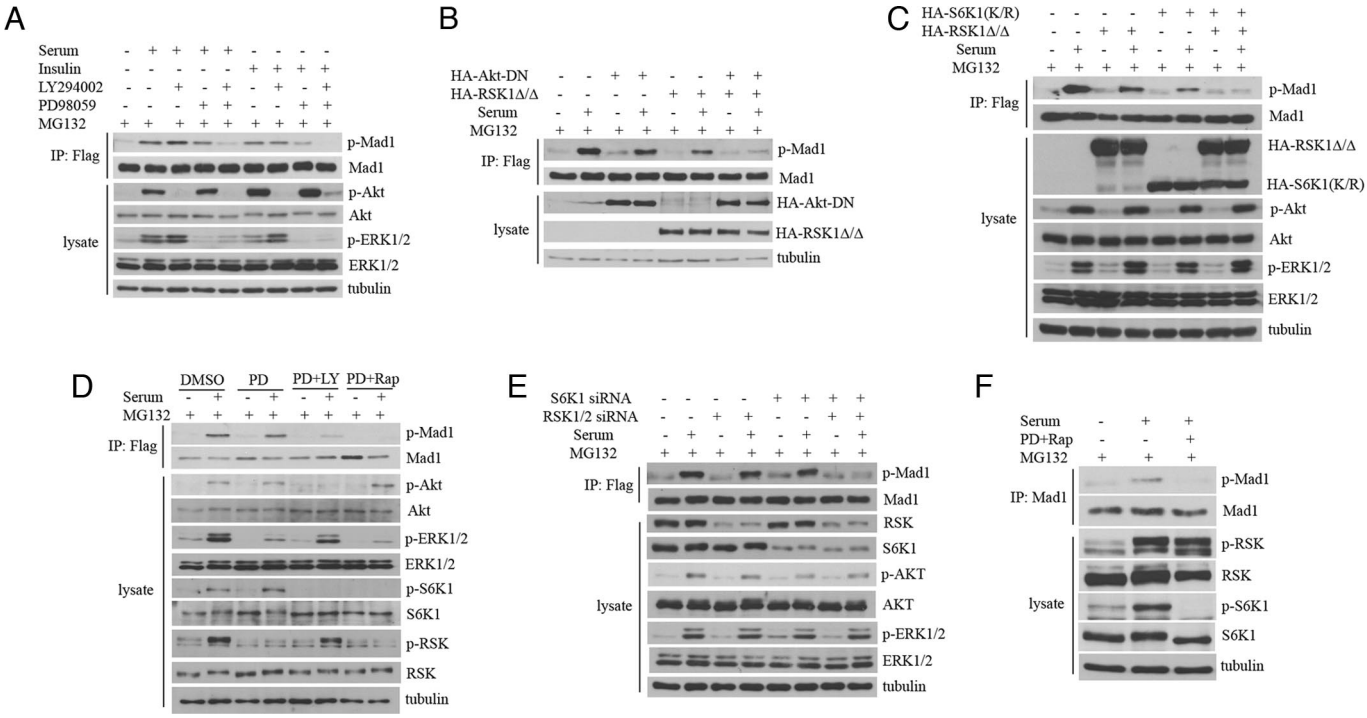

Fig. 3. Mad1 is phosphorylated by endogenous RSK and S6K1 *in vivo* upon serum stimulation. (*A*) HeLa-Flag-Mad1 cells were starved for 18 h, pretreated with MG132 (10 μM) and with LY294002 (50 μM) and/or PD98059 (50 μM) as indicated for 1 h before stimulation with serum or insulin (10 μg/ml) for 15 min. Flag-Mad1 was immunoprecipitated with anti-Flag antibody and immunoblotted with anti-phospho-Mad1 and anti-Mad1 antibodies. Total cell extracts were analyzed by Western blotting with the indicated antibodies. (*B*) 293T cells were transfected with vectors of Flag-Mad1 and kinase-dead Akt (Akt-DN) and/or kinase-dead RSK1 (RSK1Δ/Δ). The cells were serum-starved for 18 h, pretreated with MG132 (10 μM) for 1 h and stimulated with serum for 15 min. Analysis was performed as in *A*. (*C*) The experiment was performed as in *B* except that the cells were transfected with kinase-dead S6K1 (K/R) as indicated. (*D*) The experiment was performed as in *A* except with rapamycin (20 nM) as indicated. (*E*) HeLa-Flag-Mad1 cells were transfected with siRNAs targeting S6K1 and/or RSK1/2 as indicated. The total amount of siRNA was normalized by addition of a nontarget siRNA. Forty hours after transfection, cells were starved for 18 h, pretreated with MG132 (10 μM), and stimulated with serum for 15 min. Analysis was performed as in *A*. (*F*) HeLa cells ($\approx 10^8$) were starved, pretreated, and stimulated as in *A*. Mad1 was immunoprecipitated by using anti-Mad1 antibody. The immunoprecipitates and cell lysates were analyzed by Western blotting with the indicated antibodies. PD, PD98059; LY, LY 294004; Rap, rapamycin.

to an increase in basal phosphorylation of Mad1 compared with control overexpression of a kinase-inactive S6K1 (containing a K100R mutation) in the presence of proteasome inhibitor MG132. The phosphorylation was increased by insulin (0.5 μg/ml, a weak stimulator of MAPK pathway at this dose) treatment and was rapamycin-sensitive. Overexpression of an activated allele of S6K1 (F5A-E389-R3A) led to constitutive phosphorylation of Mad1 that was rapamycin-insensitive. To test whether RSK can phosphorylate Mad1 in cells, we coexpressed RSK1 with Mad1 in 293T cells, which resulted in a dramatic increase in phosphorylation of Mad1 upon stimulation with PMA (Fig. 2*E*). Moreover, when coexpressed in cells, kinase-inactive RSK1 (RSK1Δ/Δ) inhibited Mad1 phosphorylation (Fig. 2*E*). The PMA-induced Mad1 phosphorylation was inhibited by the MEK inhibitor PD98059 (Fig. 2*F*). Moreover, the mobility shift of Mad1 induced by serum stimulation was completely inhibited by S145A mutation [supporting information (SI) Fig. S1], suggesting that Ser-145 is the major, if not the only, phosphorylation site induced by serum stimulation. Overexpression of activated Akt also phosphorylated Mad1 in 293T cells (data not shown). These results suggested that these AGC kinases could phosphorylate Mad1 *in vitro* and in cells under overexpression conditions.

Mad1 is Phosphorylated by Endogenous RSK and S6K1 *in Vivo* upon Serum Stimulation. Because Mad1 is degraded in cells upon mitogen or insulin stimulation (Fig. 1), we tested whether Ser-145 of Mad1 was also phosphorylated under these conditions. Because of the low expression levels of Mad1 protein in all of the cell lines that we have tested and because our phospho-S145Mad1 antibody was not sensitive enough to detect phosphorylation of endogenous Mad1 in routine Western blot analysis, we first used a HeLa-Flag-Mad1 stable cell line to investigate the regulation of Mad1 phosphorylation. As shown in Fig. 3*A*, Ser-145 of Flag-tagged Mad1 was indeed phosphorylated upon serum or insulin (10 μg/ml) stimulation in the presence of proteasome inhibitor MG132. Consistent with the role of AGC kinases in mediating Ser-145 phosphorylation of Mad1 *in vitro*, phosphorylation of Flag-tagged Mad1 in HeLa cells was also inhibited by the combination of PI3K inhibitor LY294002 and MEK inhibitor PD98059. Interestingly, treatment of LY294002 or PD98059 alone was not sufficient to inhibit the Ser-145 phosphorylation of Mad1. This result suggests that both ERK/RSK and PI3K/Akt signaling modules are involved in Ser-145 phosphorylation of Mad1. Consistent with this proposal, serum stimulation-induced Mad1 Ser-145 phosphorylation was prevented only by the expression of both dominant negative mutants of Akt (Akt-DN) and RSK1 (RSK1Δ/Δ). Expression of either inactive Akt or inactive RSK alone, however, was not sufficient to block the phosphorylation of Ser-145 Mad1 (Fig. 3*B*), suggesting that Akt and RSK may act in a redundant fashion on Mad1 phosphorylation.

S6K1 is a downstream effector kinase of Akt upon serum or insulin stimulation, and is phosphorylated and activated in a rapamycin-sensitive manner by mTOR (13). S6K1 activation is also mediated by the Ras pathway via RSK-dependent phos-

phorylation and inactivation of the Rheb GTPase-activating protein TSC2 (14). To investigate whether Mad1 phosphorylation by RSK or Akt is mediated by S6K1, we coexpressed Mad1 with the kinase-inactive S6K1 (S6K1K/R) and/or kinase-inactive RSK1 (RSK1Δ/Δ) in 293T cells. Expression of inactive RSK1 or inactive S6K1 alone cannot inhibit Ser-145 Mad1 phosphorylation (Fig. 3*C*). However, coexpression of inactive RSK1 and inactive S6K1 together, which had no effect on the activation of Akt upon serum stimulation, totally prevented Mad1 phosphorylation (Fig. 3*C*). This result suggests that both RSK and S6K1 can independently phosphorylate Mad1 after serum stimulation. On the other hand, although Akt can phosphorylate Mad1 *in vitro* (Fig. 2*C*), activated Akt is not sufficient to mediate Ser-145 phosphorylation of Mad1 (Fig. 3*C*), and thus Akt is not likely to directly phosphorylate Mad1 in cells. To further confirm this point, we treated HeLa-Flag-Mad1 cells with PD98059 and rapamycin before serum stimulation. Consistent with the result described in Fig. 3*C*, the combination of PD98059 and rapamycin inhibited the activation of S6K1 and RSK and Ser-145 phosphorylation of Mad1, without affecting the activation of Akt (Fig. 3*D*), whereas rapamycin alone is not sufficient to inhibit Mad1 phosphorylation induced by serum (Fig. S2). Finally, using siRNA, we found that knockdown of both RSK1/2 and S6K1 is required for inhibiting S145 phosphorylation of Mad1 (Fig. 3*E*). These results suggest that both endogenous RSK and S6K can directly phosphorylate Mad1 on Ser-145 upon serum stimulation, whereas Akt is likely not a physiological kinase for Mad1.

To examine whether Ser-145 of endogenous Mad1 is phosphorylated, we immunoprecipitated Mad1 in lysate prepared from ≈10^8 HeLa cells. We found that anti-phospho-S145Mad1 recognized Mad1 in the immunoprecipitates from the cells stimulated with serum, but not in those from unstimulated cells or cells treated with PD98059 and rapamycin (Fig. 3*F*). This provides further support for our hypothesis that both endogenous RSK and S6K1 may mediate Ser-145 phosphorylation of endogenous Mad1 in cells in response to serum stimulation.

Ser-145 Phosphorylation of Mad1 Promotes Serum-Induced Mad1 Degradation. We next tested whether phosphorylation of Mad1 at Ser-145 was important for Mad1 degradation induced by serum stimulation. As shown in Fig. 4*A*, serum stimulation quickly induced polyubiquitination of Mad1, corresponding to the appearance of Mad1 phosphorylation at Ser-145. Furthermore, the treatment of PD98059 and rapamycin together inhibited both phosphorylation and ubiquitination of Mad1 (Fig. 4*B*), suggesting that Ser-145 phosphorylation of Mad1 is important for its degradation. To further test this hypothesis, we stimulated mitogen-deprived HeLa-Flag-Mad1 cells with serum in the presence of PI3K and/or MEK inhibitors. Although LY294002 or PD98059 alone could not inhibit the degradation of Mad1, the combination of these two inhibitors efficiently prevented the proteolysis of Mad1 (Fig. 4*C*).

To determine the effect of S145 phosphorylation on the stability of Mad1, we next analyzed the levels of S145A mutant Mad1 after serum stimulation. We generated two polyclonal stable HeLa cell lines expressing wild-type or S145A mutant Mad1 by infecting HeLa cells with virus expressing wild-type or S145A mutant Mad1 and selecting the survival cells as a pool. Cells stably expressing wild-type or S145A Mad1 were deprived of serum for 24 h and then restimulated with serum. The cell lysates were harvested for analysis after different periods of serum stimulation. The level of wild-type Mad1 was significantly decreased within 30 min, whereas that of S145A mutant Mad1 remained unchanged upon serum addition (Fig. 4*D*). These data indicate that Ser-145 phosphorylation of Mad1 is required for serum-induced Mad1 degradation. To further confirm this, we coexpressed Mad1 with activated S6K1 or wild-type RSK1 in 293T cells. Overexpression of S6K1 or RSK1 dramatically reduced the level of wild-type Mad1, but not that of S145A mutant Mad1 (Fig. 4*E*), suggesting that S6K1 or RSK promoted Mad1 degradation by Mad1 phosphorylation at Ser-145.

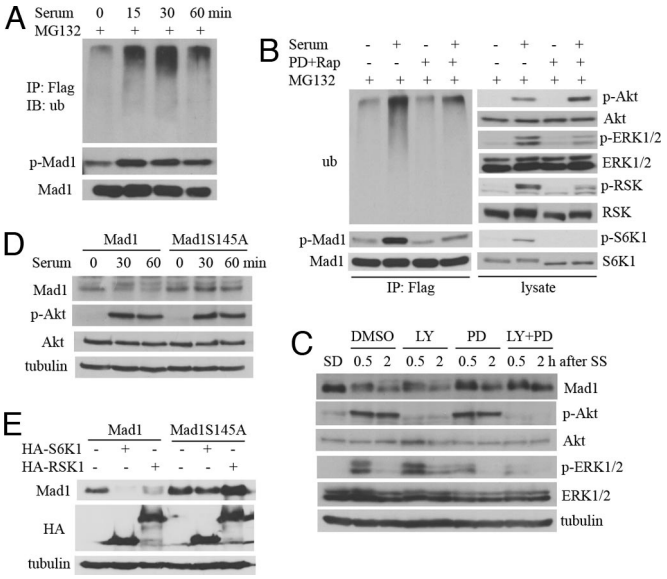

Fig. 4. Ser-145 phosphorylation of Mad1 promotes serum-induced Mad1 degradation. (*A*) HeLa-Flag-Mad1 cells were serum-starved for 18 h, pretreated with MG132 (10 μM) for 1 h, and stimulated with serum for indicated amount of time. Flag-Mad1 was immunoprecipitated and eluted with Flag peptide. The elution was analyzed by Western blotting with the indicated antibodies. (*B*) The experiment was performed as in *A* except that cells were pretreated with MG132 and with or without a combination of PD98059 (50 μM) and rapamycin (20 nM). The immunoprecipitates and lysates were analyzed by Western blotting with the indicated antibodies. (*C*) HeLa-Flag-Mad1 cells were starved (SD) for 24 h, pretreated with various drugs as indicated for 1 h, and stimulated with serum (SS) for the indicated times. The lysates were analyzed by Western blotting with the indicated antibodies. (*D*) HeLa cells expressing wild-type or S145A mutant Mad1 were starved in serum-free medium overnight. The cells were then stimulated with serum for the indicated amount of time. The lysates were analyzed by Western blotting with the indicated antibodies. (*E*) 293T cells were transfected with vectors encoding wild-type or S145A mutant Mad1 and S6K1 or RSK1 as indicated. After 24 h, cells were lysed and the lysates were analyzed by Western blotting with the indicated antibodies. LY, LY294002; PD, PD98059; Rap, rapamycin.

We have reported that c-IAP1 is the E3 ligase for Mad1 and promotes Mad1 ubiquitination and degradation (12). To test whether c-IAP1 is mediated in this serum-induced Mad1 degradation, we knocked down c-IAP1 in HeLa-Flag-Mad1 cells. Serum stimulation induced Mad1 ubiquitination to a similar extent in both control RNAi and c-IAP1 RNAi cells (Fig. S3). These results indicated that the degradation of Mad1 induced by mitogen stimulation is independent of c-IAP1.

S145A Mad1 Mutant is More Stable than Wild-Type Mad1. To further explore the significance of Ser-145 phosphorylation, we used retroviral infection to generate stable MCF10A and HeLa cells expressing wild-type or S145A mutant Mad1. Consistent with a destabilizing effect of Ser-145 phosphorylation, S145A Mad1 mutant showed a higher level of expression than that of wild-type Mad1 in both MCF10A and HeLa stable cell lines (Fig. 5*A* and data not shown). However, the mRNA levels of wild-type and mutant Mad1 were not significantly different, ruling out the possible effects of differential transcription or mRNA stability (Fig. 5*A*). To confirm that the reduced wild-type Mad1 protein levels were due to accelerated protein degradation, we used cycloheximide (CHX) to inhibit *de novo* protein synthesis in HeLa-Flag-Mad1 stable cells. Wild-type Mad1 was degraded

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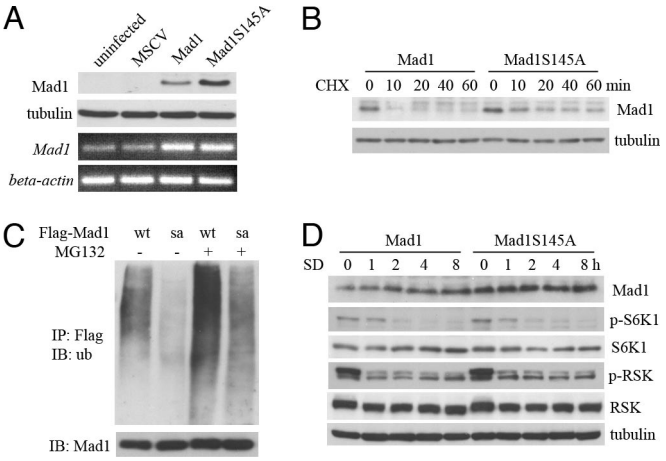

Fig. 5. S145A Mad1 mutant is more stable than wild-type Mad1. (*A*) Lysates were prepared from MCF10A cells stably expressing empty vector (MSCV), wild-type Mad1, or S145A mutant Mad1 and analyzed by using Western blotting with the indicated antibodies. The mRNA levels of Mad1 and β-actin were analyzed by RT-PCR. (*B*) HeLa cells expressing Mad1 or S145A mutant Mad1 were treated with cycloheximide (CHX; 100 ng/ml) and harvested at the indicated time points. The cell lysates were analyzed by Western blotting with anti-Mad1 and anti-tubulin antibodies. (*C*) 293T cells were transfected with vectors encoding Flag-tagged Mad1 (wt) or S145A mutant Mad1 (sa) and HA-tagged ubiquitin for 20 h and then treated with or without MG132 (10 μM) for 4 h before harvesting. Anti-Flag immunoprecipitates were analyzed by immunoblotting with the indicated antibodies. (*D*) HeLa cells expressing Mad1 or S145A mutant Mad1 were starved with serum-free medium (SD) and harvested at indicated time points. The cell lysates were analyzed by Western blotting with the indicated antibodies.

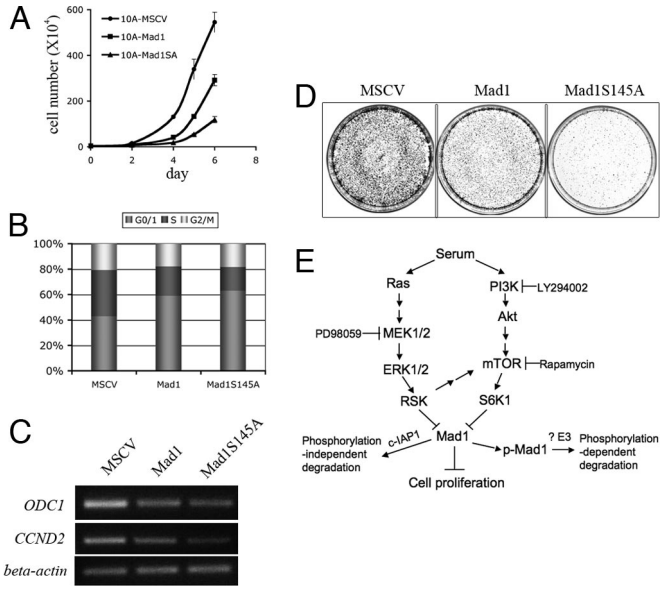

Fig. 6. Phosphorylation of Mad1 by RSK and S6K1 promotes cell proliferation and Myc-mediated transcription. (*A*) Equal numbers of MCF10A cells stably expressing indicated proteins were seeded, and cell proliferation was measured by counting cell number with a Levy hemacytometer. Data are presented as the mean ± SD of three independent experiments. (*B*) The cell cycle distributions of MCF10A cells expressing vector (MSCV), wild-type Mad1, or S145A mutant Mad1 were analyzed by FACS. (*C*) The total RNA from MCF10A cells stably expressing empty vector, wild-type Mad1, or S145A mutant Mad1 were extracted and analyzed by semiquantitative RT-PCR for the levels of *ODC1* or *CCND2* mRNAs. *β-actin* was used as an internal control. (*D*) MCF7 stable cells expressing empty vector, wild-type Mad1, or S145A mutant Mad1 were seeded at equal numbers (5×10^4 cells per plate) and cultured for 12 days. The colonies were stained with crystal violet. The experiment was repeated three times, and the representative images were shown. (*E*) Signaling pathways involved in Mad1 degradation. Activated RSK and S6K1 leads to Mad1 degradation in a phosphorylation-dependent manner, whereas c-IAP1 promotes phosphorylation-independent ubiquitination and degradation of Mad1.

quickly, with an estimated half-life of <10 min; on the other hand, although the levels of S145A Mad1 mutant dropped ≈50% upon CHX treatment for 10 min, the remaining Mad1 pool was stable after CHX treatment for 60 min (Fig. 5*B*). These results suggest that the degradation of Mad1 may be regulated by both phosphorylation-dependent and phosphorylation-independent mechanisms.

To investigate whether ubiquitination of S145A Mad1 mutant was reduced compared to that of wild-type Mad1, we coexpressed wild-type or S145A Mad1 with ubiquitin in 293T cells. The polyubiquitination of S145A Mad1 mutant was significantly reduced in both the presence and absence of MG132 compared to that of wild-type Mad1, indicating that phosphorylation of Mad1 at Ser-145 promotes Mad1 ubiquitination (Fig. 5*C*). In contrast to that of up-regulation of endogenous Mad1 after serum starvation (Fig. 1*E*), the levels of mutant Mad1 were unchanged by serum starvation (Fig. 5*D*), indicating that Ser-145 phosphorylation of Mad1 is a key regulatory event in response to mitogen stimulation.

Phosphorylation of Mad1 by RSK and S6K1 Promotes Cell Proliferation and Myc-Mediated Transcription. The expression of Mad1 negatively regulates Myc-mediated tumorigenesis by inhibiting cell proliferation (15) and transformation (16). Consistent with these reports, the expression of wild-type Mad1 in MCF10A cells led to a reduced rate of cell proliferation, whereas cells expressing S145A Mad1 mutant showed a slower growth rate than those expressing wild-type Mad1 (Fig. 6*A*). Cell cycle analysis revealed that MCF10A cells expressing mutant Mad1 had a larger population of G_1-arrested cells and fewer S phase cells than those expressing wild-type Mad1 (Fig. 6*B*).

The transcription of *ornithine decarboxylase-1* (*ODC1*) and *Cyclin D2* (*CCND2*), two Myc-responsive genes, is repressed by overexpression of Mad1 (17, 18). Consistent with these reports, we found that the mRNA levels of *ODC1* and *CCND2* were decreased in cells stably expressing Mad1. Importantly, the repression by Mad1 was further enhanced in cells stably expressing the S145A Mad1 mutant (Fig. 6*C*).

Activation of Myc promotes cellular transformation, whereas overexpression of Mad1 suppresses Myc/Ras-mediated transformation (16). To examine whether phosphorylation of Mad1 at Ser-145 can affect the ability of Mad1 to suppress transformation, we tested the efficiency of foci formation in cells expressing wild-type and S145A Mad1. MCF7 cells were infected with retrovirus encoding vector alone, wild-type Mad1, or S145A Mad1 mutant. After selection, cells were cultured for 12 days to allow foci formation. MCF7 cells expressing wild-type Mad1 showed a modest reduction in the number of foci compared to that of control stable cells, whereas S145A Mad1-expressing cells formed a dramatic reduction in foci formation (Fig. 6*D*). Taken together, these results indicate that by phosphorylating Ser-145 of Mad1 and promoting Mad1 degradation, RSK and S6K1 alleviate the repression of Mad1 on its target genes to promote cell proliferation and transformation.

Discussion

The results presented here suggest that both PI3K/Akt/mTOR and MAPK pathways regulate the Myc/Max/Mad1 network by phosphorylating Mad1 and promoting its degradation (Fig. 6*E*). Moreover, we show that S145A mutant Mad1 has an elevated effect on inhibiting cell proliferation and transformation. These

findings provide a new strategy to antagonize Myc transcription activity by inhibiting both PI3K/Akt/mTOR and MAPK pathways.

Overexpression or amplification of the *myc* gene has been detected in numerous solid tumors and blood malignancies (19). In addition, earlier studies showed that Myc protein is required for transformation by the oncogenic tyrosine kinases, including BCR-Abl (20, 21) and Src (22). Expression of nonreceptor tyrosine kinase v-Src results in tyrosine phosphorylation of numerous substrates and activation of multiple signaling pathways. Several studies showed that although inhibition of Ras/MAPK kinase pathway by expression of a dominant-negative mutant of Ras was not sufficient to prevent transformation by v-Src (23, 24), simultaneous inhibition of signaling by the Ras/MAPK pathway and the PI3K-mTOR pathway essentially blocked v-Src-induced transformation (25). These results are consistent with our hypothesis that inhibition of both the Ras/MAPK and PI3K/mTOR pathways is required to stabilize Mad1 protein and thereby antagonize Myc transcription activity.

Mad1 is a very short-lived protein, suggesting its level is tightly regulated in cells. We have identified c-IAP1 as the E3 ligase for Mad1. Overexpression of c-IAP1 decreases the level of Mad1 and thus facilitates Myc-induced transformation (12). However, in this study we found that c-IAP1 is not required for Mad1 degradation induced by serum or insulin stimulation, suggesting that multiple mechanisms exist in cells to regulate Mad1 level (Fig. 6*E*). Consistent with this possibility, we found that ≈50% S145A mutant Mad1 was still degraded quickly upon cycloheximide treatment, whereas the remaining pool of S145A mutant persists for a long time (Fig. 5*B*). Interestingly, although c-IAP1 is not required for Mad1 degradation induced by serum stimulation, our data suggest that inhibition of c-IAP1 may contribute to the increase of Mad1 levels after starvation because the binding between c-IAP1 and Mad1 is weakened after starvation (data not shown), suggesting that Mad1 is stabilized during starvation by keeping it away from its E3s, including c-IAP1.

Taken together, our study provides a mechanism to link between the growth factor signaling pathways regulated by PI3 kinase/Akt and MAP kinases with Myc-mediated transcription. Identification of the molecular mechanism that regulates the protein levels of Mad1 provides a new strategy to suppress Myc-mediated cellular transformation and tumorigenesis by inhibiting both RSK and S6K1.

Materials and Methods

siRNAs. siRNA sequences targeting RSK1, RSK2, and S6K1 (Qiagen) are 5′-TGCCACGTACTCCGCACTCAA-3′, 5′-CCGAGTGAGATCGAAGATGGA-3′, and 5′-GGGAGTTGGACCATATGAACT-3′, respectively.

Antibodies. The following antibodies were used: anti-Mad1 C19 (Santa Cruz Biotechnology); anti-Akt, anti-phospho-Akt (Ser-473), anti-ERK1/2, anti-phospho-ERK1/2 (Thr-202/Tyr-204), anti-p90RSK, anti-phospho-p90RSK (Ser-380), anti-S6K1, and anti-phospho-S6K1(Thr-389) (Cell Signaling Technology); anti-HA (Covance); anti-ubiquitin (DAKO); anti-Flag M2 and anti-tubulin (Sigma). Phospho-Mad1 antibody was raised against peptide ERIRMD-pS-IGSTVSS and was custom-made by Proteintech Group.

***In Vitro* Kinase Assay.** GST fusion proteins (1 μg) were incubated with recombinant active Akt (50 ng, Upstate Biotechnology) or RSK1 (50 ng, Echelon Biosciences) in 25 μl of kinase buffer [8 mM Mops/NaOH (pH 7.0), 0.2 mM EDTA, 10 mM MgAc and 0.1 mM ATP] at 30°C for 30 min in the presence of 5 μCi [γ-^{32}P]ATP. The reaction products were resolved by SDS/PAGE and ^{32}P-labeled proteins visualized by autoradiography.

Foci Formation Assay. MCF7 stable cells were seeded to 6-cm diameter culture plates (5×10^4 cells per plate) and then cultured for 12 days. Cells were washed with cold PBS and fixed with methanol at 4°C for 10 min. Colonies were stained with crystal violet.

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